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13. ABSTRACT (Maximum 200 words)
During this period of activity, we published our first comprehensive journal article from results in this project: see Proceedings of the National Academy of Sciences 92:397-401, 1995 (attached). A no-cost extension of the project was approved to extend activities to 31 Aug '96. At the ECU performance site, four trials of hemostatic efficacy of our rehydrated lyophilized platelet preparations were carried out in live pigs according to the Letterman Army Institute animal model of hemorrhagic shock. The results were quite variable and showed the need to adapt this model to emphasize the role of platelets and control other physiologic parameters related to the effects of hypovolemic shock. Attempts in vitro to load rehydrated platelets with calcium ion flux indicator dyes were finally successful, and studies are now underway to measure the responsiveness of these platelet preparations to thrombin and other agonists that rely on $Ca^{2+}$ for signal transduction. Experiments conducted at UNC showed serotonin uptake and release by rehydrated platelets and surface-related changes in activation. It will be important to continue to define the extent of activation and its control in our rehydrated lyophilized platelet preparations.

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Dear Sir,

Please find enclosed two copies of the Second TriAnnual Report (Year 2) for the period January 1, 1995 - April 30, 1995. If you have any questions I may be contacted at 919-816-5020. Thank you.

Arthur P. Bode, Ph.D.
Principal Investigator

APB/bab

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SECOND TRIANNUAL REPORT (YEAR 2)

for period January 1, 1995 to April 30, 1995

Report Date: July 5, 1995

ONR Grant No. N00014-93-J-1034
(ECU Grant #5-01071)

# PRECLINICAL INVESTIGATION OF LYOPHILIZED PLATELET PREPARATIONS

Principal Investigator: Arthur P. Bode, Ph.D.
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Attachments:

1. Report from subcontract principal investigator, Marjorie S. Read, Ph.D., The University of North Carolina at Chapel Hill.
2. Publication in Proc. Natl. Acad. Sci., and editorial commentary.

Administrative Activity:

This reporting period included the annual program meeting and review at the Naval Research Laboratory in Alexandria, VA, on April 20. At that review was presented a summary of current data on the animal models in which our efforts have intensified over the past four months. On learning of our difficult start with the Letterman aortotomy swine model, CAPTs Weinberg and CDR June suggested that further testing be suspended until discussions are held with CAPT Charles Gray in San Diego who has developed an alternative similar model. These talks are underway as of the writing of this report and should lead to a resumption of activity in a few weeks.

The first joint ECU/UNC-CH publication on the lyophilized platelet project was published in Proceedings of the National Academy of Sciences (USA): Volume 92, pp. 397-401 (see attached). This article was the subject of the editorial commentary of that issue, reflecting its great potential impact on laboratory medicine. Another paper submitted in November to the journal BLOOD came back without acceptance, but is undergoing rewriting for resubmission. Also during this period, we have been seeking to extend the project into new areas to continue the work towards its final product: another set of specific aims has been approved by the ATD after scientific review, but no start date has yet been given. On April 19, another patent application relevant to the pharmaceutical production of lyophilized platelets was filed in the U.S. Patent and Trademark Office with inventors from Armour and Drs. Bode and Read. This particular protection was sought for the antimicrobial effects of using 1.8% paraformaldehyde to stabilize the platelets prior to freeze-drying. Armour and UNC-CH produced data showing bacterial and viricidal potential with 1.8% para and consider it possible to apply to future blood products such as freeze-dried RBC. Although it may appear to be tangential to the specific aims of this project, demonstrating sterility of the pharmaceutical product is very important to the successful application of this work to military medicine.

Both ECU and UNC-CH have experienced reductions in technical workforce in our research laboratories, partly due to the finish of N00014-92-J-1244, and also due to seasonal attrition at our universities. We have requested a no-cost one year extension of the termination date of current project activity under N00014-93-I-1034 from Aug. 31, 1995 to Aug. 31, 1996 to accommodate a somewhat slowed pace of experimentation. This will be necessary to complete the contemplated animal model studies demonstrating hemostatic efficacy and low immunogenicity of the platelets in circulation. Approximately $240,000 of the grant remains unspent at the time of this report to be carried over into the extension period. Of that, $45,000 is considered frozen until new animal study protocols have been approved by the ECU IRB and ONR.

Scientific Progress:

At ECU our main effort in this reporting period has been in the production and testing of lyophilized platelet preparations from pigs and dogs in collaboration with co-investigators in the Dept. of Surgery. Several canine preps that were not used in earlier

experimentation in the heart-lung bypass model were analyzed after as much as 9 months storage freeze-dried at -70°C. Microscopic examination showed that the earlier preps had worse Kunicki morphology scores, and the most recent had the best scores (up from 155 to a high of 253). This finding probably does not reflect just a deterioration in physical state of the platelets during storage, but rather continuing improvements in preparation techniques (such as introduction of the use of PGE-1 as a preservative) in the later attempts. Yields upon reconstitution also were better in the later preps. We think that this evidence of improvement in preparations suggests that further dog studies should be carried out in the heart-lung bypass model to check for better quality of response in the hemostatic markers after infusion. The range of Kunicki morphology scores in the pig lyophilized platelet preparations has ranged from 180 to 220, and all are used in the fresh state (storage for < 1 month). A few of the unused preparations will be set aside for 9 months for similar analysis of storage stability; these preps did not have significant changes in technique and should address the stability question better than the canine preps.

We have conducted four trials of the Letterman aortotomy model in pigs at ECU, using packed RBC from donor pigs and freshly-prepared (< 1 month storage) lyophilized platelets. In the model, a 5 mm tear is introduced into the infrarenal aorta of the animal subject via a wire stitch threaded and pulled through the abdominal wall. The rapid loss of blood is abated only because of a tamponade effect as the extravascular hematoma accumulates in the retroperitoneum. The intent is to produce a hemorrhagic shock state over the next two hours and test the effect of platelet infusions on the prevention of exsanguination when surgical repair of the aorta is performed. Small, 25 kg swing were selected as the surgical subjects, while larger pigs were used as donors of up to 6 units of blood during the week before for preparation of packed RBC and lyophilized platelets to be used in the surgical animals.

In the first trial, the pig tolerated the initial injury without demise and showed some evidence of the predictable hemorrhagic shock state. Repeated RBC and reconstituted platelets infusions during and after surgical repair showed a reversal of the loss of hemostatic function in the animal as reflected in the ACT and readings from the Clot Signature Analyzer instrument measuring circulating platelet function. We concluded that these preliminary findings demonstrated good hemostatic efficacy of the platelet preparations, but we did not have a measure of the impact of infusions on secondary bleeding sites nor was it obvious when in the course of events that infusion of platelets would have the greatest effect. The second trial included a modification in that several sutures were placed in apical portions of the lungs to provide a secondary injury site from which to assess small vessel bleeding complications caused by loss of platelet count and function in the shock state. Unfortunately, when these stitches were pulled shortly after the aortotomy, copious blood flow clogged the respiratory ventilation line and the pig was lost before infusion data could be collected. The third animal subject survived both the aortotomy and the secondary (reduced) wound in the lung initially, but expired just prior to the platelet infusions and surgical repair due to an unintentionally prolonged shock development stage (3 hours instead of 2). The fourth trial was conducted much more

attentively, but the pig did not appear to enter a state of profound shock. The platelet count did not drop below 200,000/$\mu$L, the hematocrit stayed above 20, and the Clot Signature Analyzer showed the onset of a hypercoagulable state but did not become prolonged as in shock, the fibrinogen level stated between 150 - 250 mg%, and the drainage from the lung injury never increased beyond the first 40 mls to indicate rebleeding. In spite of these non-critical findings, we proceeded with RBC and platelet infusions after attempting to re-injure the aorta and then effect repair. There was some evidence of hemostatic effect of the platelet infusions from the CSA data, but a more profound injury state would have provided a better setting for assessment. Attached to this report are brief data and event summaries from the first and fourth trials in this model. We hope to refine and continue this model after discussion of possible modifications and extensions of the research plan with CAPT Charles Gray at the Clinical Investigation Department of the Naval Medical Center at San Diego.

Both UNC-CH and ECU have been carrying out further in vitro characterization of reconstituted human lyophilized platelets to assess the integrity of the activation response machinery upon physiologic stimulation. See Dr. Read's attached subcontract report for data on serotonin uptake/release and characterization of other activation byproducts. At ECU, we have implemented a series of flow cytometry studies to demonstrate cytoplasmic calcium ion flux in fresh, stored, and reconstituted lyophilized platelets. It has taken some time to find indicator dyes that load into rehydrated platelets without leaking or excessive binding to the platelet surface. The optimal system appears to be one using fluo3 and fura red in a radiometric analysis in gel-filtered platelets. We had tried previously using Indo-1 or aequorin without obtaining useful signals because of loading problems (reported under N00014-92-J-1244). At present, we have recorded a 10 - 60 second spike in $Ca^{2+}$ flux in fresh and stored blood bank platelets stimulated with Thrombin that is nearly as large as that seen with calcium ionophore. With reconstituted lyophilized platelets, we have recorded in preliminary experiments a similar reproducible $Ca^{2+}$ flux in response to ionophore, even without external $Ca^{2+}$ in the medium buffer (evidence of activation of internal $Ca^{2+}$ stores in these platelets). Early experiments suggest that it may take a 10-20 fold increase in Thrombin concentration to stimulate a $Ca^{2+}$ flux response in rehydrated platelets to equal that of fresh platelets. More data will be presented in the next reporting period as more preparations of platelets are tested under the now refined conditions.

The next phase of project activity will continue with the dog and pig hemostasis models, the biochemical analysis of activation responses in rehydrated platelets, and studies of their immunogenicity and circulatory capacity in non-surgical models. We foresee a successful conclusion to each specific aim if granted the no-cost extension of the project period to August, 1996.

Triannual Report January 1, 1995 - April 31, 1995
University of North Carolina at Chapel Hill

Contract: UNC/ECU
Grant No. N0014-93-1034
The Office of Naval Research
Department of the Navy

Performance Site: University of North Carolina at Chapel Hill
Principal Investigator: Marjorie S. Read, Ph.D.
Submitted: June 7, 1994

1. Do platelets exposed to paraformaldehyde retain metabolic function? One function of platelets is the uptake and storage of plasma serotonin. The ability of platelets to take up plasma serotonin is due to a specific transport system modulated by some platelet membrane components such as membrane receptors. Serotonin is implicated in platelet/vessel wall interactions and in blood pressure rise. In some studies (human recombinant erythropoetin studies) it has been suggested that platelet serotonin correlates well with normal bleeding time. Uptake of serotonin is one test of metabolic function of platelets. In that light we have studied the uptake and release of serotonin in fixed-rehydrated platelets.

In our previous report we examined the serotonin release mechanism of rehydrated platelets using a fluorometric assay. Using that assay we were unable to reliably measure serotonin uptake. Since then, we have used a radioisotope procedure to measure serotonin uptake. Rehydrated platelets take up about 40% as much serotonin as fresh platelets. The variability seen with the assays appears to be related to the separation of bound and unbound isotope on a sepharose column. We achieved better separation of platelets and free serotonin using a micro-filtration technique in which free serotonin was passed through the filter and the platelets were retained.

Table 1.

**$^{14}C$-Serotonin Uptake**

| Assay # | % Serotonin Uptake by platelets | | |
|---|---|---|---|
| | Fresh | Rehydrated | Rehydrated/ Fresh |
| 1 | 57.6 | -- | -- |
| 2 | 23.6 | 2.90 | 12.30 |
| 3 | --- | 1.5 | --- |
| 4 | --- | 2.2 | --- |
| 5 | --- | 16.5 | --- |
| 6 | 37.15 | 15.46 | 41.6 |
| 7 | 28.75 | 10.7 | 37.2 |

Assay#1 through #4= platelets labeled with $^{14}C$ serotonin were separated on a sepharose column.

Assays #5 through #7 platelets labeled with $^{14}C$ were separated with a 0.22 micron filtration system.

All data in Table 1 above were collected from studies performed at 37$^0$C.

The rehydrated platelets took up serotonin at about 40% the amount of that taken up by fresh platelets. That is consistent with other studies performed in Dr. Bode's laboratory showing reduced, but definite response in other metabolic response tests such as hypertonic shock and arachidonic acid metabolite production.

2. Biochemical characterization of rehydrated platelets. In order to evaluate changes induced in rehydrated platelets by the processing we have used two methods.

(1) A bank of antibodies were used to compare fresh and rehydrated platelets for the presence of antigens common to platelets. Platelets were treated or not treated with triton to perforate the membrane and allow antibody to enter the platelet. We compared fresh and rehydrated platelets in an effort to detect neo-antigens in or on the surface of rehydrated platelets and to determine if internal antigens had been lost during processing. Studies were performed with fresh and fixed platelets spread on glass for 15 -20 min at room temperature. Spread platelets were treated with triton, washed, blocked and incubated with antibody to proteins being studied. Bound antibody was detected with a fluorescent secondary antibody. Staining patterns for rehydrated and fresh platelets were evaluated. Surface staining was more uniform with bright surface staining. Internal proteins caused a stippled or punctate staining. The results are shown in Table 2.

Table 2.

| Antibodies used: | Staining Results (Thrombin Stimulated) | | | |
|---|---|---|---|---|
| | Fresh | | Rehydrated | |
| | Internal | Membrane | Internal | Membrane |
| Serotonin | + | ++ | + | ++ |
| GPlb | + | ++ | + | ++ |
| GPllb/llla | + | + | + | + |
| fibrinogen | ++ | +++ | ++ | + |
| fibronectin | ++ | + | +/- | +/- |
| vWF | ++ | + | + | +/- |

In the absence of thrombin, no surface serotonin was detected on either fresh or fixed platelets. Both fresh and fixed platelets contained serotonin by several different assays. Greater release from fresh than fixed platelets was noted. Brighter staining with fixed, supports more internal, unreleased serotonin in fixed platelets.

With GPlb antibodies, There was no difference in the staining pattern on fresh and fixed platelets with and without thrombin stimulation.

With antibodies against llb/llla, without thrombin stimulation, staining patterns were similar for both fresh and fixed platelets. With thrombin, there appeared to be a decrease of surface staining with fixed platelets. This is consistent with decreased fibrinogen binding seen in other studies presently being conducted. Whether thrombin cleaves the receptor from fixed platelets, an unlikely result, or whether the decreased staining is the result of lack of exposure of membrane receptors by thrombin is not clear at this time.

Anti-fibrinogen antibodies indicated more internal fibrinogen in both fixed and fresh platelets in the absence of thrombin stimulation. Following thrombin stimulation, there was brighter staining indicating more external, surface fibrinogen.

Fresh platelets stain brighter for fibronectin, internal and external with and without thrombin stimulation.

There is little surface staining for vWF with fixed platelets. Thrombin stimulation makes no difference. There is internal staining as seen with triton treatment of fixed platelets. The fresh show some surface vWF and more internal staining.

(2). The same bank of antibodies was used to evaluate and compare proteins released from fresh and fixed platelets. Platelets were incubated in buffer for 30-60 min at 37°C. The platelets were removed and the supernate tested by ELISA to identify antigens released from fixed and fresh platelets. Platelets were also lysed with triton and the total protein lysate tested by ELISA.

Results: See enclosed figures 1 and 2.

Fig. 1. Releasate from human fresh (Fr) and fixed (Fx) platelets. Release of fibrinogen, thrombospondin and fibronectin are markedly greater for fr than fx platelets. All others appear about the same.

Fig. 2. Comparison of amount of protein released from fixed platelets to the amount obtained from lysed fixed platelets. The release of

granule proteins from fixed platelets appears to be nearly complete as compared to the lysates.

SDS gel electrophoresis of released material from fresh and fixed platelets were also performed and the proteins were detected with silver staining. Some of the high molecular weight bands (consistent with vWF) were not present or were reduced with fixed platelets. Most other bands were present. Fig. 3 is a copy of photos showing PAGE electrophoresis of fresh and fixed platelet secreted material.

These data from spread platelets, ELISA assays and SDS gel electrophoresis, analysis of released material from fresh and fixed platelets indicated that there are some differences between fresh and fixed platelets. So far no neo-antigens have been found with fixed platelets. The difference between fixed and fresh platelets appears to be one of degree, not a total change or completely altered cell.

The biochemical and immunologic classification of fixed platelets is ongoing in our laboratory. To date we feel the findings are very favorable. It is imperative, however, that these studies continue to make human trials a real possibility.

Fig. 1

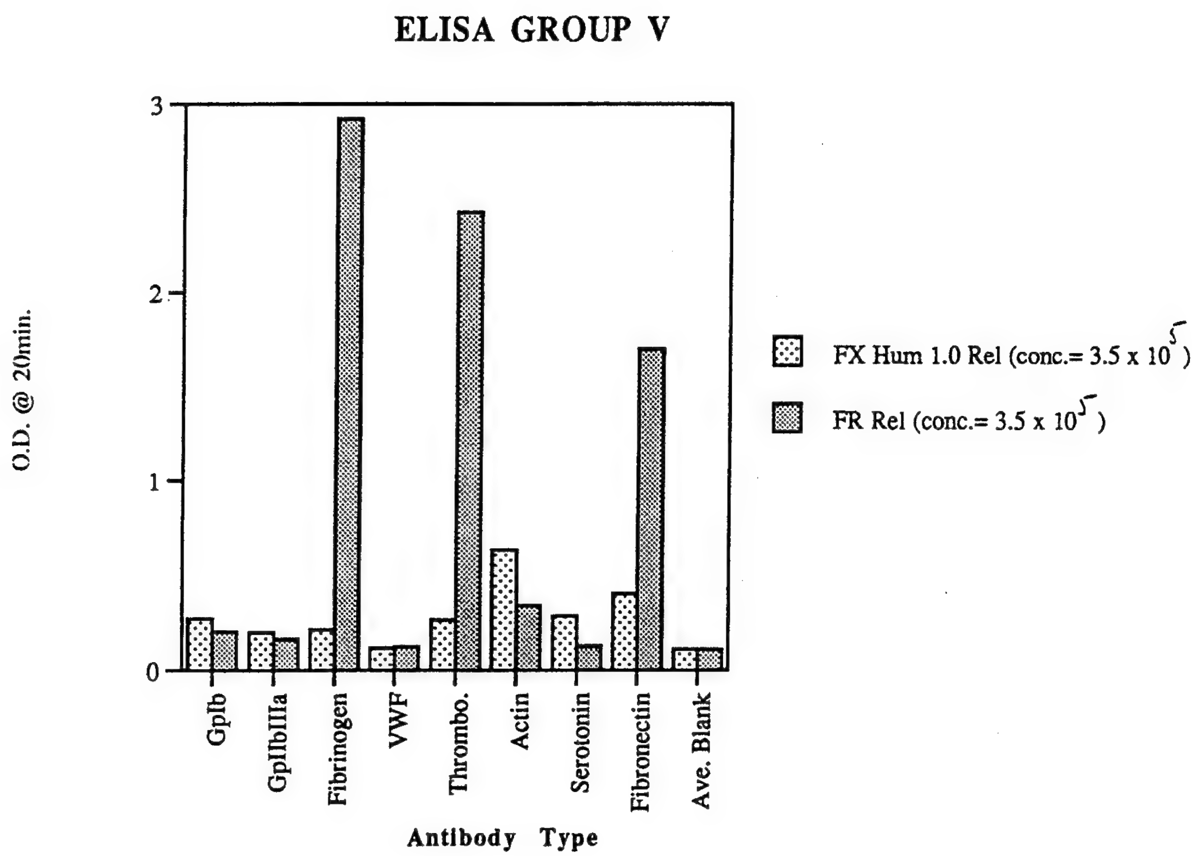

ELISA GROUP V
O.D. @ 20min.
3
2
1
0
GpIb
GpIIbIIIa
Fibrinogen
VWF
Thrombo.
Actin
Serotonin
Fibronectin
Ave. Blank
Antibody Type
FX Hum 1.0 Rel (conc.= 3.5 x 10^5)
FR Rel (conc.= 3.5 x 10^5)

Fig. 2

ELISA GROUP III

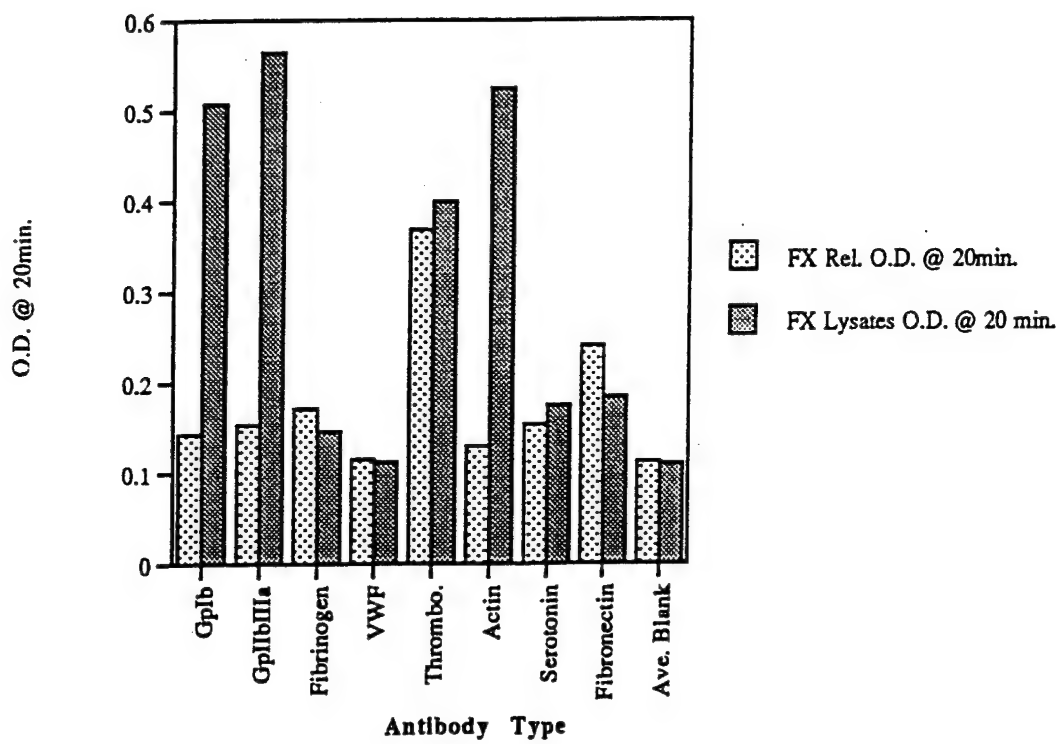
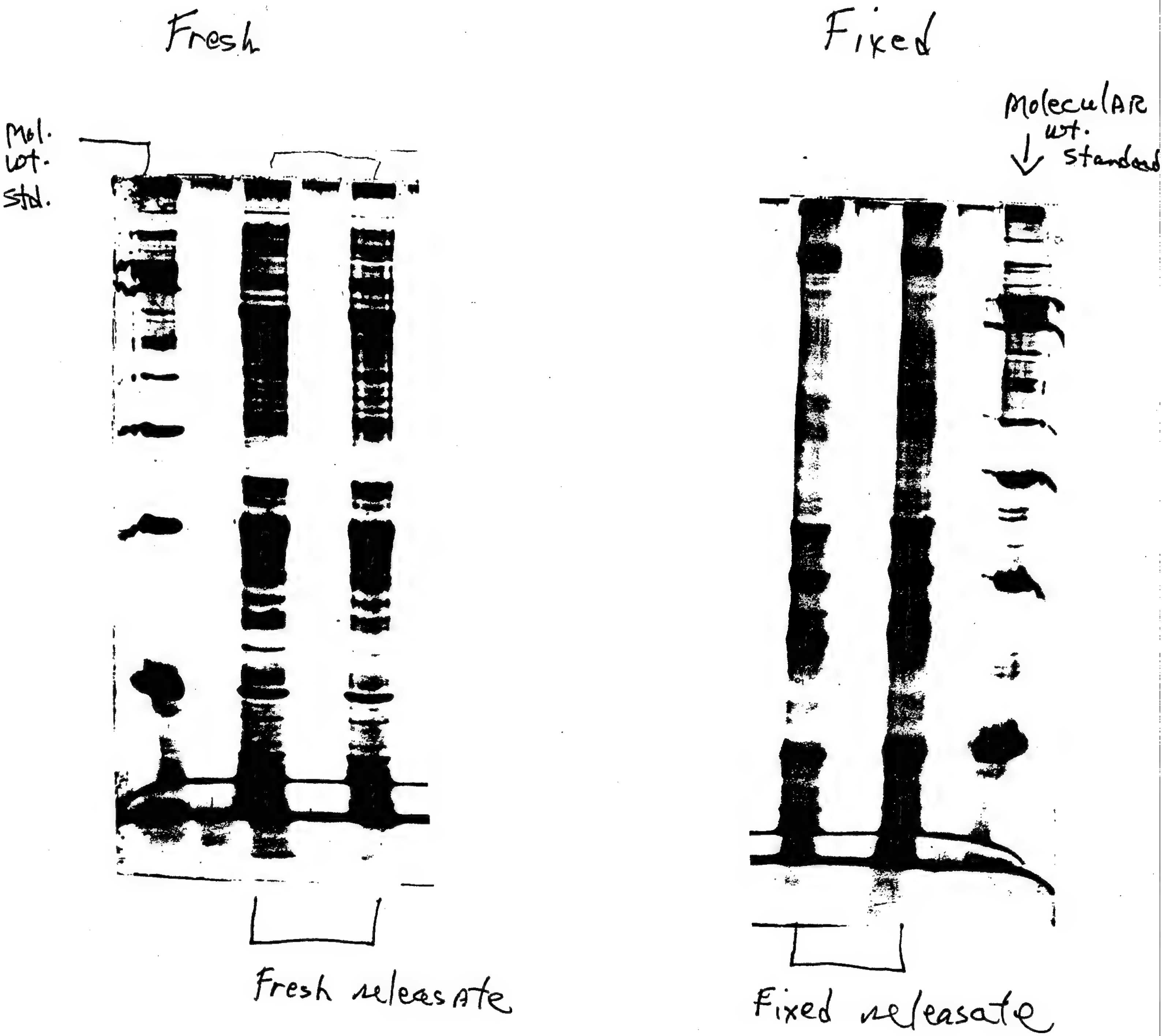


Fig. 3


Fresh
Fixed
Mol. wt. std.
Molecular wt. standard
Fresh releasate
Fixed releasate

# PIG #1 (01/26/95) ABDOMINAL AORTA INJURY

| EVENT | HCT | ACT | CSAIVBT | CSACITF | CSACCT |
|---|---|---|---|---|---|
| 1. Presurgical baseline | 31 | 67 sec. | 10:11 sec | 100% | 8 min. |
| 2. Spleen out, wire in aorta | 26 | 63 sec. | | | |
| ----------INJURY INDUCED (11:20)---------- | | | | | |
| 3. 20 min. post-injury | 17 | 52 sec. | | | |
| ----------RBC INFUSION (12:15)---------- | | | | | |
| 4. 5 min. post RBC-infusion<br>One hour post-injury | 27 | 67 sec. | ~0:10 sec. | 100% | 8 min. |
| 5. Two hours post-injury | 17 | | | | |
| ----------RBC & REHYDRATED PLT INFUSION (1:30)---------- | | | | | |
| 6. One min. post-infusion<br>2 1/4 hrs post-injury;<br>during injury repair | 27 | 88-106 sec. | 2:10 | 1% | 19 min. |
| ----------RBC & REHYDRATED PLT INFUSION (2:00)---------- | | | | | |
| 7. One min. post-infusion<br>2 3/4 hrs post-injury | 29 | 178 sec. | 5:29 | 1% | 22 min. |
| 8. 30 min. post-infusion<br>three hours post-injury | 34 | 122 sec. | | | |
| ----------REHYDRATED PLT INFUSION (2:50)---------- | | | | | |
| 9. 30 min. post-infusion<br>4 hrs post-injury<br>(1 1/2 hrs post-repair) | 35 | 110 sec. | 3:22 | 33% | 12 min. |

# PIG #4 (03/30/95) ABDOMINAL AORTA INJURY

| EVENT | PLTS | HCT | ACT | FIBG | CSAIVBT | CSACT1 | CSACCT |
|---|---|---|---|---|---|---|---|
| 1. Presurgical baseline | | | 100 sec. | 195 | | | |
| 2. Spleen out, wire in aorta | | 38 | 91 sec. | | 5:15 | 20:04 | 22:00 |
| ----INJURY INDUCED (10:20)---- | | | | | | | |
| 3. 5 min. post-injury | 419 | 32 | 69 sec. | 148 | | | |
| 4. 20 min. post-injury | | 31 | 67 sec. | 197 | 1:36 | 11:36 | 11:00 |
| 5. 40 min. post-injury | | 31 | 60 sec. | | | | |
| 6. 60 min. post-injury | | 31 | 66 sec. | 242 | CLOTTED IN SYRINGE | | |
| 7. 90 min. post-injury | ~~[illegible]~~ 401 | 29 | 53 sec. | | | | |
| ----REINJURY (12:10)---- | | | | | | | |
| 8. 2 hrs post-injury | 354 | 26 | 72 sec.. | 262 | CLOTTED IN SYRINGE | | |
| ----REOPEN ABDOMEN AND INJURY (12:45)---- | | | | | | | |
| 9. 10 min. post new injury | | 25 | 83 sec. | | | | |
| 10. 30 min post new injury | | 24 | 63 sec. | | | | |
| 11. 45 min. post new injury | 306 | 24 | 61 sec. | 205 | CLOTTED IN SYRINGE | | |
| ----RBC (ONE UNIT) THEN LYOPHILIZED PLTS (100 X $10^9$) (2:10)---- | | | | | | | |
| 12. 5 min. post-infusion 90 mins. post new injury | 235 | 36 | 80 sec. | 124 | 2:48 | 11:12 | 9:45 |
| ----LYOPHILIZED PLTS (106 X $10^9$) INFUSED (2:40)---- | | | | | | | |
| 13. 5 min. post 2nd infs 2 hrs post new injury | 233 | 35 | 71 sec. | 188 | 0:59 | 8:42 | 8:00 |
| 14. 40 min. post 2nd inf 2 1/2 hrs post new injury | 240 | 37 | 78 sec. | 171 | 0:40 | 7:19 | 7:00 |

Proc. Natl. Acad. Sci. USA
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Medical Sciences

# Preservation of hemostatic and structural properties of rehydrated lyophilized platelets: Potential for long-term storage of dried platelets for transfusion

(platelet adhesion/platelet agglutination/thrombocytopenia/thrombosis)

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*Contributed by Kenneth M. Brinkhous, September 22, 1994*

**ABSTRACT** **Currently, therapeutic platelet concentrates can be stored for only 5 days. We have developed a procedure that permits long-term storage of fixed and lyophilized platelets that retain hemostatic properties after rehydration. These rehydrated lyophilized platelets (RL platelets) restore hemostasis in thrombocytopenic rats and become incorporated in the hemostatic plug of bleeding time wounds of normal dogs as well as von Willebrand disease dogs with partially replenished plasma von Willebrand factor. Ultrastructurally, these platelets are well preserved and are comparable to control normal washed platelets. Flow cytometry analysis shows that RL platelets react with antibodies to the major surface receptors, glycoprotein (GP)Ib and GPIIb/IIIa. These receptors are involved in platelet agglutination, aggregation, and adhesion. *In vitro* functional tests document the ability of RL platelets to adhere to denuded subendothelium and to spread on a foreign surface. Circulating RL platelets participated in carotid arterial thrombus formation induced in normal canine subjects. The participation of RL platelets in these vital hemostatic properties suggests that with further development they could become a stable platelet product for transfusion.**

To promote effective hemostasis, platelets must respond quickly to changes in normal blood flow or vessel injury (1, 2). After vascular injury, platelets adhere to exposed subendothelium, aggregate, and form a primary platelet plug. Platelet activation and initiation of coagulation follow with stabilization of the platelet plug by the formation of fibrin. The initiation of a thrombus at a site of vascular injury is mediated through platelet membrane glycoprotein (GP) receptors (3, 4). Platelet adhesion to a damaged vessel wall and its extracellular matrix at high shear is primarily mediated through the specific interaction of the platelet membrane GPIb–IX complex and bound von Willebrand factor (vWF) (5–7), which is synthesized and released into plasma and the vessel wall by endothelial cells (1). Platelet adhesion at low shear rates is mediated by several interactions, including collagen with the $\alpha_2\beta_1$ integrin (7). Platelet adhesion stimulates a spreading of the platelet (8). Although the mechanism of platelet spreading has not been completely characterized, recent *in vitro* studies have shown that platelets will spread on surfaces coated with fibrinogen (9) or polymerized fibrin (10). The activation of the GPIIb/IIIa receptor by agents such as ADP results in a conformational change in the receptor (11–13). The activated receptor binds fibrinogen, which forms a "bridge" between the platelets, and causes aggregation (1, 14, 15). Activated platelets provide the phospholipid surface for the assembly of blood clotting enzyme complexes, and the concentration and localization of activated coagulant proteins at sites of vessel wall injury may be facilitated by adherent platelets (16). Internal storage granules in platelets release clot-promoting contents in response to activation of biochemical systems triggered by platelet–platelet or other interactions. Interactions of adherent platelets with neutrophils, mediated through platelet integrins, specifically P-selectin receptor (17), may contribute to hemostatic and other cell functions (13).

The control of hemorrhage due to thrombocytopenia often requires transfusion of multiple units of fresh platelets. In transfusion medicine, platelets cannot be replaced by other blood products or artificial media. Maintenance of critical membrane GPs during storage is crucial to platelet function *in vivo*. With the storage life of fresh platelets limited to 5 days, there has been considerable study to lengthen platelet shelf life and enhance stored platelet response (18–24). Investigators have addressed storage conditions that preserve platelet integrity and responsiveness (25, 26). The effects of preservatives on platelet activation and expression of membrane GPs have also been investigated (27). In a recent review, the use of inhibitors of platelet activation to extend the shelf life and enhance the quality of liquid stored platelets is discussed (28). Cryopreservation of platelets extends the shelf life to 1 year but requires extensive washing and processing to remove cryoprotectant agents (29). In other blood cell studies, red blood cells washed with saline followed by lyophilization retain metabolic activities similar to red blood cells stored under blood bank conditions (30). Lyophilization of platelets or platelet-rich plasma as previously attempted (31–33) neither preserved the structural integrity of the platelets nor provided adequate hemostasis when infused into thrombocytopenic pediatric patients or hemorrhagic animal models.

We have successfully prepared a paraformaldehyde-treated, lyophilized and rehydrated platelet product (RL platelets) with intact morphology and agglutinating properties (34–36). Efforts to refine this process have produced platelet preparations that are structurally stable and capable of undergoing activation. We report here on the hemostatic properties displayed by our RL platelets as tested *in vitro* and in canine and rat animal models. In this study, we have administered RL platelets to normal and von Willebrand disease (vWD) dogs and to thrombocytopenic rats. Our results indicate that RL platelets retain many essential biologic properties and promote hemostasis. The successful preparation of a dried transfusion platelet product without loss of hemostatic capabilities

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Abbreviations: RL platelet, rehydrated lyophilized platelet; BT, bleeding time; vWD, von Willebrand disease; vWF, von Willebrand factor; GP, glycoprotein.

‡To whom reprint requests should be addressed.

suggests potential for the development of this product as a blood banking resource.

## MATERIALS AND METHODS

**Preparation of RL Platelets.** Lyophilized human and canine platelets were prepared as described (34). Human platelets were obtained from the American Red Cross 3–6 days after collection. Canine platelets were obtained from normal dogs ($n = 5$) from the Francis Owen Blood Research Laboratory (University of North Carolina). Washed platelets were incubated for 1 hr with paraformaldehyde at concentrations of 1.8% for human platelets and 0.68% for canine platelets. Washed paraformaldehyde-free platelets in citrated saline (0.006 M trisodium citrate/0.154 M NaCl, pH 6.8) with 5% bovine serum albumin were frozen in 1-ml aliquots containing $8 \times 10^8$ platelets per ml and lyophilized at −20°C to −40°C for 20–24 hr. Dried platelets were stored at −80°C until used. Dried platelets were rehydrated in 1.0 ml of imidazole buffer (IB; 0.084 M imidazole, pH 7.35) and centrifuged at 1000 × *g* for 8 min to pellet the platelets. The rehydrated platelets were freed of albumin and imidazole by three washes in citrated saline. For use, the platelet pellets were resuspended in platelet-poor plasma or in a modified Hanks' buffered salt solution (mHBSS; 0.17 M NaCl/6.7 mM KCl/1.0 mM $MgSO_4$/0.5 mM $K_2HPO_4$/2.8 mM $Na_2HPO_4$/13.8 mM dextrose, pH to 7.2 with 1.4% $NaHCO_3$) for *in vitro* studies and in normal saline for *in vivo* studies. Gas chromatography was used to document the absence of formaldehyde in washed canine and human RL platelet solutions. A detection limit of 0.002% was used (National Medical Laboratories, Willow Grove, PA).

RL platelets were labeled with the fluorescent dye Zynaxis PKH 26 as a marker for platelets in infusion studies. The pelleted rehydrated platelets were washed once by resuspension in 1.0 ml of acid citrate dextrose, centrifuged at 600 × *g* for 8 min, and resuspended in 0.1 ml of mHBSS. The mixture was incubated for 20 min in the dark and centrifuged (600 × *g*; 8 min). The labeled platelets were washed once by resuspending in 1.0 ml of mHBSS containing either 0.1% canine serum albumin or 0.1% bovine serum albumin. No label was transferred or lost from fluorescent platelets incubated at 25°C–37°C for several hours in whole blood.

**Hemostatic and Other Methods.** The saline bleeding time (BT) in canines was performed as described (37). The BT wound sites were excised and prepared for fluorescence and light microscopy (38). For the rat toenail BT, rats were anesthetized with ketamine hydrochloride (Ketaset)/ProMACE, and a foot was antiseptically cleansed and warmed in a 37°C bath. A sterile scalpel blade was used to excise the distal 1.0 mm of the vascular nail bed from one nail. Blood was blotted onto filter paper for BT measurements. Platelet adhesion of fresh and RL human platelets was compared in an annular perfusion chamber (39). Adhesion studies were carried out at high shear (flow rate, 125 ml/min; 37°C) using porcine arterial subendothelium that had been denuded by exposure to air. After platelet solutions containing fresh or RL platelets were exposed to the subendothelium, segments were removed and processed for scanning electron microscopy to visualize platelet adhesion. Citrated blood, platelet-rich plasma, platelet-free plasma (PFP), and red blood cell fractions were isolated as described (34). Adhesion of fresh platelets was determined after passing whole citrated blood over the subendothelium. Adhesion of RL platelets was determined after PFP enriched with RL platelets and the red blood cell fraction was passed over the subendothelium. To confirm the absence of platelets in PFP prior to the addition of RL platelets, phase-contrast microscopy was used. To examine platelet spread, RL platelets were reconstituted in IB, washed once in HBSS (40) to remove albumin, and spread on Formvar-coated grids (41). Spread platelets were examined with a Cambridge autoscan scanning electron microscope at 20 kV.

For morphological studies, fresh and RL platelet pellets were processed for transmission electron microscopy as described (38). Platelets were examined with a Zeiss 10A microscope. Rehydrated platelet surface antigen distribution and overall light scatter properties were analyzed on a Becton Dickinson FACS 440 flow cytometer. Monoclonal antibody binding to rehydrated platelets or to fresh platelets resuspended in citrated plasma was evaluated by indirect immunofluorescence 488-nm excitation as described (42). Control antibody was used to identify nonspecific IgG binding to fresh and rehydrated platelets. In each run, 10,000 events were measured and analyzed.

**Infusions of RL Platelets.** The animals used were normal dogs ($n = 3$), a vWD dog ($n = 1$), and Sprague–Dawley rats ($n = 3$). The vWD dog was from the closed colony at the Francis Owen Blood Research Laboratory. Normal Sprague–Dawley rats were obtained from the Division of Laboratory Animal Medicine (University of North Carolina, Chapel Hill). All animals were treated according to published standards (43). Thrombocytopenic rats were given $4.5 \times 10^9$ and $3.4 \times 10^{10}$ human RL platelets, respectively, through the tail vein. Toenail BTs were performed immediately after infusion of RL platelets. Normalization of BT was taken as an indicator of RL platelet hemostatic function.

Fluorescence-labeled canine RL platelets were infused into three normal dogs. After infusion of these platelets, plasma levels of vWF (44), coagulation factor IX (45), coagulation factor VIII (46), platelet counts (Unopette; Becton Dickinson), and serum fibrin degradation products (Thrombo-Wellcotest; Wellcome) were determined prior to and at the

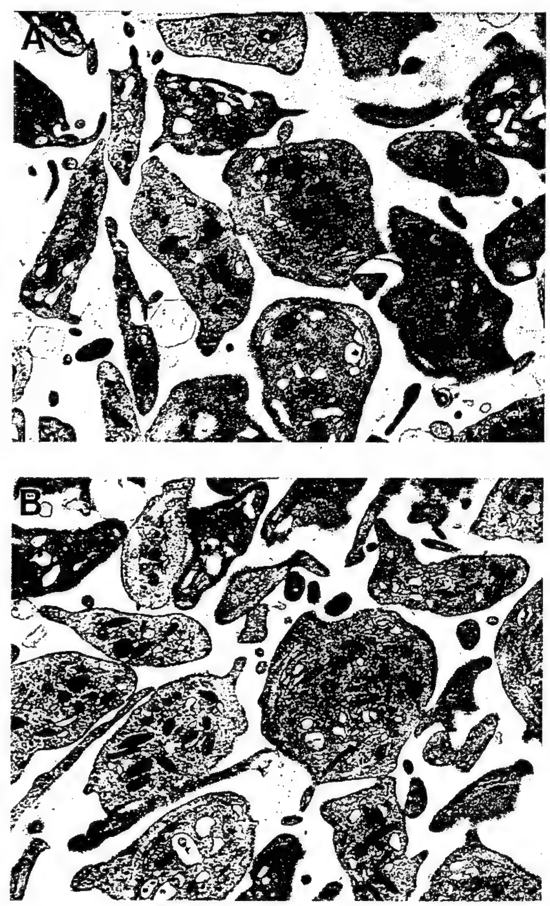


FIG. 1. Transmission electron microscopy of fresh (*A*) and RL (*B*) platelets. Both fresh and RL platelets have intact and randomly distributed organelles and some pseudopod formation. (×7760.)

Table 1. GPIb, GPIIb/IIIa, and GPIb/IX on the surface of fresh and RL platelets

| Antigen | Antibody | % labeled platelets | |
|---|---|---|---|
| | | Fresh | RL |
| GPIb | AN-51 | 98 | 92 |
| GPIb | SZ-2 | 92 | 85 |
| GPIb/IX | SZ-1 | 98 | 92 |
| GPIIb/IIIa | 10ES | 98 | 98 |

Monoclonal antibodies to GPIb (clones AN-51 and SZ-2) were obtained from Dakopatts (Glostrup, Denmark) and AMAC (Westbrook, ME), respectively. The anti-GPIb/IX complex antibody (SZ-1) and the anti-GPIIb/IIIa complex antibody (clone 10E5) were obtained from AMAC and Barry Coller (State University of New York, Stony Brook), respectively. There were <5% labeled platelets in the positive gates using a control nonimmune mouse IgG-2a antibody (Coulter Immunology). Fresh platelets were washed with citrated saline.

following intervals postinfusion: 1, 5, 15, 30, and 60 min during the first hour, and 2, 4, 6, 8, and 24 hr thereafter. BTs were performed postinfusion of labeled RL platelets and wounds were excised after cessation of bleeding for examination by fluorescence and light microscopy (38). A similar infusion was performed with a vWD dog after partial replacement of vWF by treatment with cryoprecipitate. RL platelets represented 51.4% of the vWD dog's normal platelet count based on the number of RL platelets infused. Cryoprecipitate was prepared as described (47) and assayed for vWF content (44). A modified Folts procedure (48) was used to produce carotid arterial thrombosis in dogs ($n$ = 3).

## RESULTS AND DISCUSSION

**Structural Features of RL Platelets.** Transmission electron microscopy showed that RL platelets are morphologically similar to fresh washed platelets (Fig. 1). RL platelets are partially activated, similar to fresh washed platelets. Flow cytometry using anti-GPIb and anti-GPIIb/IIIa monoclonal antibodies indicated that both GPs were present on the surface of RL platelets (Table 1). The number of RL platelets with antibody recognition of receptors is expressed as a percentage of the platelets with specific immunofluorescence.

**Functional Characteristics of RL Platelets *in Vitro*.** Platelet adhesion and platelet spreading are shown in Fig. 2. A comparison of platelet adhesion with fresh and RL platelets shows that RL platelets adhere in numbers similar to fresh platelets, with irregular shapes and with multiple pseudopodia,

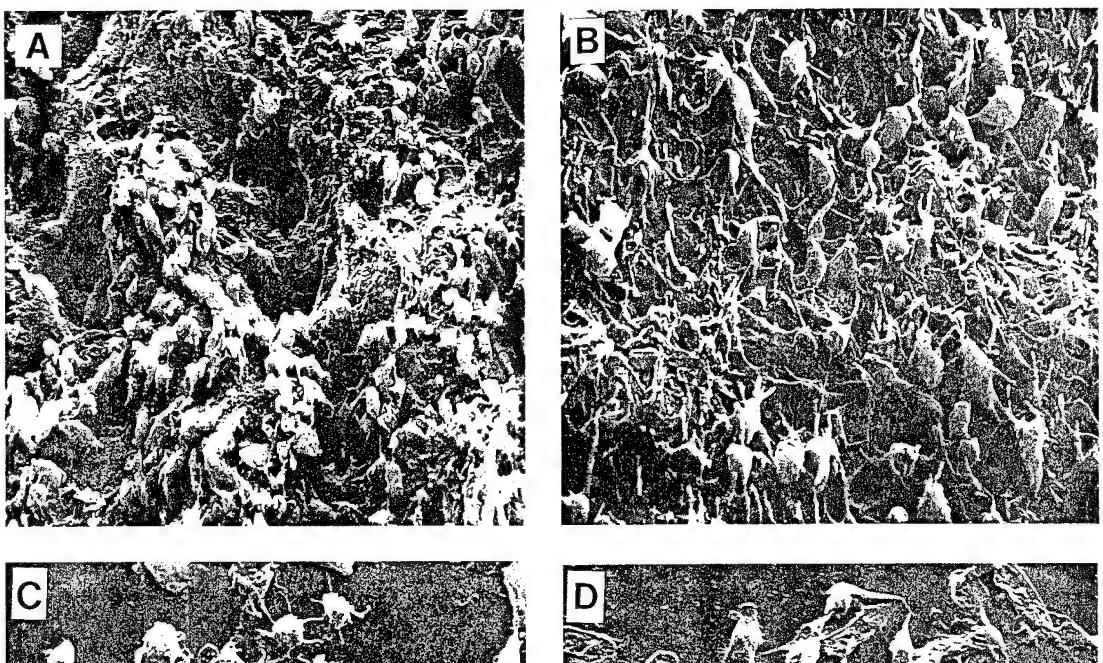



FIG. 2. Scanning electron microscopy (SEM) of vessel segments from an annular perfusion chamber with RL platelets ($A$) and fresh platelets ($B$). Vessel subendothelium exposed to platelet-free blood was free of platelets (data not shown), while segments exposed to platelet-containing blood was carpeted with platelets. SEM of spread RL platelets shows that paraformaldehyde-stabilized platelets adherent to Formvar-coated grids formed dendritic patterns with multiple pseudopodia and are fully spread ($C$), similar to fresh platelets spread on the same substrate ($D$). ($A$, ×960; $B$, ×2320; $C$, ×2080; $D$, ×1600.)

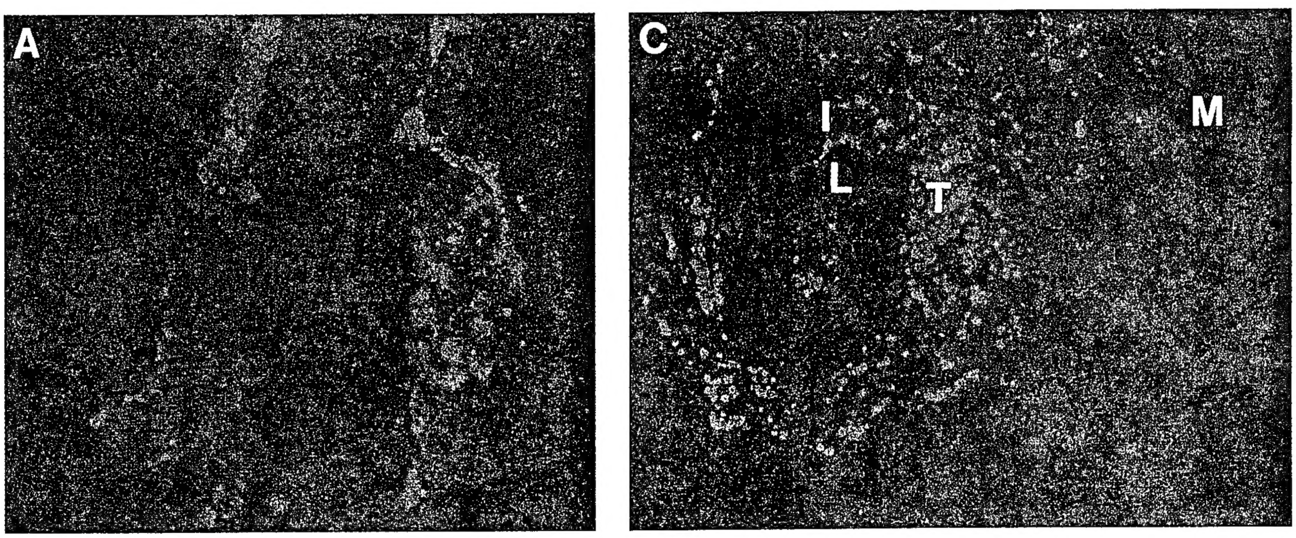
Table 2. Infusions of human RL platelets shorten the prolonged BTs in thrombocytopenic rats

| | Normal | | Thrombocytopenic | | Thrombocytopenic with RL platelets | |
|---|---|---|---|---|---|---|
| Animal | BT, min | Platelet count per $\mu$l, $\times 10^{-3}$ | BT, min | Platelet count per $\mu$l, $\times 10^{-3}$ | BT, min | Platelet count per $\mu$l, $\times 10^{-3}$ |
| 1 | 0.5 | 685 | >15 | 25 | 0.5 | 220 |
| 2 | 2.0 | 580 | >15 | 32.5 | 1.5 | 237 |

Thrombocytopenia was induced in two Sprague–Dawley rats by treatment with 1 ml of a 1:10 dilution of anti-rat thrombocyte polyclonal antibody (Accurate Chemicals). Platelet counts and toenail BT measurements were used to monitor the level of circulating rat platelets 10 min after treatment with the antibody. RL platelets were infused immediately after a BT of >15 min was established. In a control rat without treatment with RL platelets, bleeding times were >15 min, and platelet counts were <50,000 platelets per $\mu$l for >12 hr.

although pseudopodia are present to a lesser extent in RL platelets (Fig. 2 *A* and *B*). Neither RL platelets nor fresh platelets were present in areas where the endothelium remained intact (data not shown). A comparison of platelet spreading of fresh and RL platelets showed both having a similar flattened or "pancake" morphology (Fig. 2 *C* and *D*). Multiple pseudopodia were found associated with both fresh and RL platelets, which were not completely spread. Few discoid forms were present, suggesting that paraformaldehyde-stabilized platelets retained sufficient metabolic activity for platelet spreading to occur. Earlier studies have demonstrated that functional GPIb is preserved in lyophilized platelets (49). While GPIIb/IIa epitopes are identified in RL platelets, minimal platelet aggregation was observed in preliminary studies with ADP (A.P.B., unpublished data).

**Functional Characteristics of RL Platelets *in Vivo*.** RL platelets were labeled with a fluorescent dye to distinguish rehydrated platelets from circulating native platelets in infusion experiments. BT studies in rats with human RL platelets and in normal and vWD dogs with canine RL platelets were conducted. The results of two separate RL platelet infusion experiments using thrombocytopenic rats are shown in Table 2. After administration of human RL platelets, toenail BTs in two rats treated with an anti-rat thrombocyte antibody decreased from >15 min to normal. One rat was tested at 30 min and had a toenail BT of 3.5 min, which remained corrected for 1 hr, at which time the rat was sacrificed. Treatment of normal rats with diluted anti-rat thrombocyte antibody depleted circulating rat platelets to <33,000 platelets per $\mu$l and lengthened the rat toenail BT to >15 min. Normal rats treated with experimental levels of the anti-rat thrombocyte antibody without additional RL platelets had elongated BTs (>15 min) and low autologous platelet counts (<50,000 platelets per $\mu$l) for 12 hr. The toenail BT is a simple and reproducible method of measuring BT in the rat and requires only that the rat be anesthetized before testing. In 10–12 normal rats, the BTs ranged from 30 sec to 3 min.



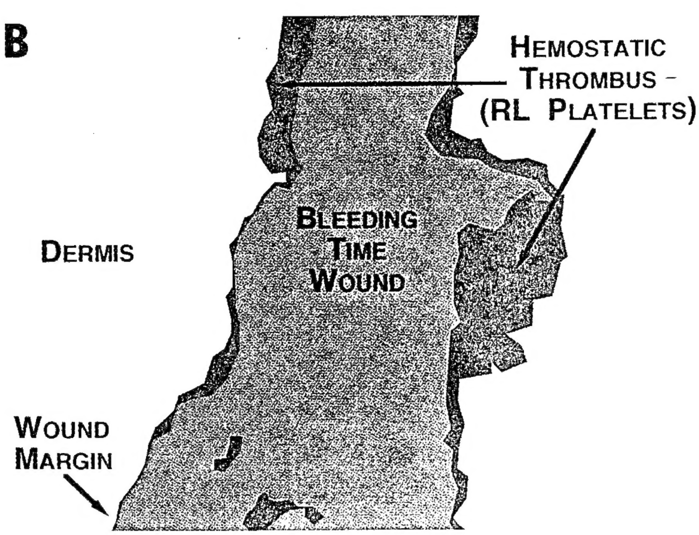




FIG. 3. Fluorescence microscopy (*A*) and a schematic representation (*B*) of an ear BT wound of a normal dog after infusion of labeled rehydrated platelets. Clumps of labeled platelets adhere to the cut surfaces of the ear wound. (*C*) Fluorescence microscopy of a carotid artery with deep vessel wall injury in a normal dog after administration of fluorescent RL canine platelets. Internal elastic lamina (I) is disrupted and deep medial injury is present. Platelet thrombi (T) in the lumen (L) and in the superficial and deep media (M) contain labeled rehydrated platelets. In these models, with a mixture of native and RL circulating platelets, RL platelets represented between 18.4% and 43.8% of the total platelet count based on the number of RL platelets infused. (×280.)

RL platelets were infused into three normal dogs and one vWD dog in order to determine whether the RL platelets were incorporated into the hemostatic thrombi of BT wounds. The vWD dog was infused with cryoprecipitate, which raised the vWF level to 38% of normal; the BT of the vWD dog was reduced from >15 min to 8 min. RL platelets were then administered to the vWD dog, and there was no significant change in the BT. The BT remained in the normal range (6 min) for the other three dogs. RL platelets circulated for the duration of the experiments (up to 4 hr). They were found to be part of the hemostatic plug in normal dogs (Fig. 3 *A* and *B*). In the vWD dog, RL platelets were observed at the same sites as in the normal dogs but in far fewer numbers (data not shown). Samples of liver, lung, and kidney were examined after sacrifice of these animals. No gross or microscopic changes were observed.

Carotid arterial thrombosis was induced by using a canine model of stenosis and injury in three normal dogs that had been infused with RL platelets (48, 50). All animals experienced occlusive thrombosis as indicated by the cessation of blood flow. When thrombosis occurred, the vessels containing the thrombi were harvested after >30 min of observation and examined by fluorescence microscopy. Fluorescent RL platelets were present in the induced thrombi and were also adherent to the exposed subendothelium. Single and aggregated platelets were present in areas of hemorrhage (Fig. 3*C*). In vessels where damage was minimal, fluorescent RL platelets were seen in the lumen and adhering to the luminal surface where the internal elastic lamina was disrupted. There was no evidence of RL platelets adhering to intact endothelium. The absence of disseminated intravascular coagulation was indicated by no change in fibrinogen level, no loss of coagulation factors VIII and IX, and no appearance of fibrin degradation products.

Platelet preparations can be stored for several days without the use of refrigeration. After this period, platelets lose many of their functions (51, 52). Lyophilization has the potential to extend blood cell (erythrocytes and platelets) shelf life from days to years. The fixation of platelets in paraformaldehyde followed by lyophilization has proven effective in maintaining some of the normal functions of the human platelet (34). We have shown that RL platelets retain many properties necessary for normal hemostasis. These observations suggest that this method of stabilization may offer a method for long-term storage of platelets.

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# Commentary

## Freeze-dried blood cells: Therapeutic advance or laboratory curiosity?

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Platelets are anucleate, cellular elements present in circulating blood that are critical for the achievement of hemostasis following tissue injury and, probably, for general maintenance of vascular integrity. The human circulation normally contains $\approx 2.5 \times 10^8$ platelets per ml, each $\approx 5\ \mu m^3$ in volume. Over the past 3 decades, much has been learned about the biochemical mechanisms and structural changes that underlie platelet function. Simply stated, platelet membranes contain a group of receptors that can be activated by substances such as ADP and epinephrine as well as by vessel wall constituents such as collagen and von Willebrand factor. These receptors are linked to biochemical pathways that transmit signals leading to structural and conformational changes in platelet membranes and membrane-associated glycoproteins. The "activated" platelets adhere to damaged vascular surfaces and to one another to form hemostatic plugs and to promote blood coagulation.

When the importance of platelets in hemostasis was recognized >80 years ago, it was natural to consider whether these cells could be transfused with therapeutic effect to patients with low platelet levels (thrombocytopenia) and hemorrhagic symptoms. This was achieved in a small number of studies (1), but the lack of effective anticoagulants and systems for collection, isolation, and storage of platelets made routine platelet transfusion therapy impractical. This situation changed in the 1950s and 1960s when the development of plastic containers and improved anticoagulants together with the recognition that platelets, in contrast to red cells, are adversely affected by refrigeration and tolerate storage best at room temperature made it possible to maintain the hemostatic effectiveness of platelets for up to 5 days prior to transfusion (2, 3).

Ready availability of stored platelets led to a remarkable increase in the utilization of this blood component for the prevention and treatment of bleeding in patients with thrombocytopenia or qualitative (functional) platelet defects. The favorable impact of platelet transfusions on medical and surgical practice is exemplified by a dramatic reduction in mortality due to hemorrhage in patients with acute leukemia since 1970 (4). In 1989, the total volume of platelets transfused to patients in the United States approximated the amount that could be derived from ≈7.5 million units of donated blood (5). This compared with ≈12.5 million units of red cells transfused. Platelet utilization has increased significantly since that time, while red cell usage has declined.

Although the quality and quantity of platelets available for transfusion today are much improved in comparison with 20 years ago, it is generally agreed that platelets stored in the liquid state for up to 5 days are less functional and survive somewhat less well in a recipient's circulation than fresh platelets (2, 3, 6, 7). Moreover, storage of platelets at ambient temperature (20–24°C) allows for the possibility of bacterial growth in occasional units, and this can have dire consequences for a transfusion recipient (8, 9). On occasion, the demand for platelets outstrips the supply, and shortages develop. Thus, there remains a need for better methods of long-term platelet storage under conditions that preserve function and viability while reducing the likelihood of bacterial growth.

More than 30 years ago, attempts were made to store platelets for long periods of time in gelatin (10) and in lyophilized form (11) with unsatisfactory or marginal results (12, 13). Subsequently, it was learned that human platelets frozen in dimethyl sulfoxide could survive and function after transfusion (14), but this method has proved impractical for large-scale application. More recently, several groups have examined whether liposomes containing platelet membranes (15) or microparticles derived from normal platelets (16, 17), preparations that could probably be stored indefinitely, are capable of producing hemostasis when transfused to thrombocytopenic patients. At this time, it is uncertain whether either of these approaches will lead to a product that can be transfused with benefit to thrombocytopenic patients.

Lyophilization (freeze-drying) is another approach to long-term preservation of blood cells for transfusion that was applied recently to red blood cells with reasonable preservation of metabolic activity following reconstitution (18). In a recent issue of this journal, Read and coworkers (19) described the application of this technique to platelets. Washed platelets isolated from human blood were fixed with paraformaldehyde, frozen in citrate buffer containing 5% albumin, freeze-dried, and stored at −80°C. They were then rehydrated and their *in vitro* and *in vivo* properties were studied. Ultrastructural integrity of the reconstituted platelets was remarkably well preserved, and they were shown to be capable of adhering to subendothelium and of spreading on an inert surface almost as well as fresh platelets. Human platelets preserved in this way, when transfused to thrombocytopenic rats, increased circulating platelet levels to a hemostatic range and shortened the bleeding times of the animals from >15 min to the normal range of 0.5–1.5 min. The reconstituted lyophilized (RL) platelets, when labeled with a fluorescent probe and transfused to three normal dogs, became incorporated into wounds inflicted on these animals by a small incision in the ear and into thrombi formed in the carotid artery following a crush injury. The stored platelets were unable, however, to aggregate normally *in vitro* after stimulation with the agonist ADP.

These studies are remarkable in that they demonstrate convincingly that lyophilized platelets can be reconstituted with some degree of preservation of function by *in vitro* and *in vivo* criteria and suggest a possible approach to large-scale platelet preservation that might overcome some of the shortcomings of existing methods. However, additional studies are needed to determine whether this technique is practical for platelet transfusion therapy. Preparation of platelets for storage by the method described is likely to be quite labor intensive and costly. Conceivably, the process could be automated, but this might require pooling of platelets from many donors to achieve a scale large enough to realize cost efficiencies. Large-scale pooling of platelets is likely to be unacceptable because of the increased risk of transfusion-transmitted viral infection when a product derived from many donors is transfused to a single recipient. Reconstitution of platelets after storage might also be time consuming and expensive. It is unknown whether paraformal-

dehyde (PFA) treatment, which appears to be important for the preservation of structure and function of the freeze-dried platelets, will affect their immunogenicity. This is more than a theoretical concern in view of the existence of naturally occurring human antibodies that recognize platelets treated with PFA (20). Finally, *in vivo* studies with RL platelets have been carried out to date only in a small number of rats and dogs. Larger numbers of animal studies and, eventually, investigations in humans will be necessary to evaluate the effectiveness of this preparation. Nonetheless, these observations are important because they suggest the possibility that platelets (and perhaps other blood cells) can be lyophilized, stored for long periods of time, reconstituted, and transfused with therapeutic benefit. Future developments in the laboratories of these investigators will be awaited with great interest by the transfusion medicine community.

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